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Autophagy Negatively Regulates Keratinocyte Inflammatory Responses via Scaffolding Protein p62/SQSTM1

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The scaffolding adaptor protein p62/SQSTM1 (p62) has been shown to be an autophagy receptor that acts as a link between the ubiquitination and autophagy machineries. However, the roles of autophagy and p62 in human keratinocytes are not well understood. In this study, we show that keratinocyte autophagy negatively regulates p62 expression, which is essential for the prevention of excessive inflammation and the induction of cathelicidin in human keratinocytes. Stimulation of TLR2/6 or TLR4 in primary human keratinocytes robustly activated autophagy pathways and up-regulated p62 expression through induction of NADPH oxidases 2 and 4 and the generation of reactive oxygen species. MyD88 and TNFR-associated factor 6, key signaling molecules that mediate TLR activation, played an essential role in the induction of autophagy and p62 expression. Additionally, blockade of autophagy significantly increased the generation of inflammatory cytokines and expression of p62 in primary human keratinocytes. Notably, silencing hp62 through RNA interference resulted in a significant decrease in NF-κB activation, inflammatory cytokine production, cathelicidin expression, and cell proliferation (as well as cyclin D1 expression) in keratinocytes. Epidermal expression of p62 was further found to be significantly higher in psoriatic skin than in skin affected by atopic dermatitis or from healthy controls. Collectively, our data provide new insights into the roles of autophagy and p62 in controlling cutaneous inflammation.


As the primary component of the epidermal barrier, keratinocytes play an essential role in innate immune responses to exogenous pathogens. TLRs are key pattern recognition molecules in innate immunity that activate the NF-κB pathway, resulting in the production of antimicrobial immune and proinflammatory mediators (1). Whereas several TLRs, including TLR2, 3, and 5, are constitutively expressed, others are inducible in human keratinocytes (2). Excessive activation of TLRs in keratinocytes has been reported in chronic inflammatory conditions, including psoriasis (3). Notably, TLR2 has been reported to be upregulated in psoriatic skin and cells from patients with psoriatic arthritis (4, 5), suggesting that it may play an important role in chronic cutaneous inflammation. Recent studies have highlighted the potential of TLR2-targeting therapies to blunt inflammatory responses without interfering with microbial recognition (6).

Autophagy is a major intracellular process in which lysosomes degrade protein aggregates, cytoplasmic organelles, and aged proteins (7, 8). Several TLR ligands, including a subset of TLR7 ligands and the TLR4 ligand LPS, are potent inducers of autophagy in macrophages (7, 9, 10). Recent studies have implicated autophagy in the regulation of inflammatory immune responses (11). The protein p62 (also known as SQSTM1) has been reported to link the recognition of polyubiquitinated protein aggregates to the autophagy machinery (12). As a signaling adaptor, p62 has been implicated in the activation of the transcription factor NF-κB through interaction with TNFR-associated factor (TRA6) (13). Additionally, p62 interacts with the autophagic effector protein LC3 and is degraded through an autophagy–lysosome pathway (14, 15). A recent study showed that p62 protein accumulates in autophagy-defective tumor cells, and that the modulation of p62 expression by autophagy is a key factor in oncogenesis (16). These data suggest that p62 accumulation associated with the dysregulation of autophagy is involved in increased inflammation and tumorigenesis. However, the role and regulation of p62 in human keratinocytes have not been characterized.

In this study, we demonstrate that stimulation of human keratinocytes with TLR2/6 ligands strongly induces p62 expression and activates autophagy through NADPH oxidase (NOX)-dependent generation of reactive oxygen species (ROS). MyD88 and TRAF6, signaling molecules that participate in TLR-dependent activation of NF-κB signaling, were necessary for the induction of p62 expression and autophagy. Moreover, inhibition of autophagy significantly increased the accumulation of p62 (which is responsible for the activation of NF-κB signaling), the expression of cathelicidin/LL-37, and the production of inflammatory cytokines. Notably, skin from patients suffering from psoriasis displayed increased p62 and cathelicidin expression, indicating that...
p62 accumulation may contribute to the pathogenesis of this chronic inflammatory skin disorder.

Materials and Methods

Cell cultures

Human primary keratinocytes and HaCaT cells were cultured and maintained, as described previously (17). Briefly, foreskin epidermal keratinocytes were cultivated in keratinocyte serum-free medium supplemented with bovine pituitary extract and recombinant human epidermal growth factor (Life Technologies/Life Technologies, Eggenstein, Germany). HaCaT cells were cultured in DMEM (Life Technologies-BRL, Gaithersburg, MD), supplemented with 10% FCS (Invitrogen, San Diego, CA), 1% L-glutamine, penicillin (50 U/L; Life Technologies-BRL), and streptomycin (50 mg/mL; Life Technologies-BRL). The study protocol was approved by the Institutional Review Board of Chungnam National University College of Medicine. Written informed consent was obtained from each participant.

Reagents, DNA, and Abs

MALP-2, a diacylated lipopeptide, was purchased from IgemGen (San Diego, CA). N-acetylcycteine (NAC), diphenylene iodonium (DPI), and allopurinol, E. coli (Escherichia coli) (Sigma-Aldrich). After 12 d, relative titers were determined by colony forming units (CFUs) in well plates, and then nontarget pLKO.1 nonspecific control shRNA (shNS) was transfected with scrambled, Small interfering RNA (siRNA) transfection was performed, as described previously (17). Briefly, HaCaT cells were transfected with scrambled, or siRNAs specific for hNOX4, hATG5, hATG7, hATG12, hATG16L1, and hATG13 (Thermo Shandon, Pittsburgh, PA). Negative control sections were treated with serum from nonimmunized rabbits (Dako) in place of primary Ab. Cells were fixed in 4% paraformaldehyde at 4˚C for 10 min. They were then permeabilized with 0.01% Triton X-100, before being blocked with 10% BSA for 1 h. Cells were stained with primary Ab (rabbit anti-human, diluted 1:400) and biotin-labeled secondary Ab (anti-rabbit IgG-tetramethylrhodamine isothiocyanate or anti-rabbit IgG Alexa488, and anti-rabbit IgG-tetramethylrhodamine isothiocyanate Abs were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The NF-κB luciferase reporter plasmid was a gift from G.M. Hur (Chungnam National University, Daejeon, Korea). A NF-κB-luc adenoviral vector (AddSVN-NF-κB-luc) was purchased from Genetron Transfer Vector Core (Iowa City, IA).

Lentiviral packaging and short hairpin RNA transduction

Human pLKO.1 lentiviral constructs from Open Biosystems, targeted to hbeclin-1 (NM_003766), hatg5 (NM_004849), and hp62/SQSTM1 (NM_001142298), were supplied by Sigma-Aldrich as glycerol stocks. Virus production was performed, as described previously (17, 18). Briefly, lentiviruses were transduced into human epithelial keratinocyte 293T cells with pLKO puro.1 or short hairpin RNA (shRNA) plasmid, and the following packaging plasmids: pMDL-RRE (5 μg), pRSV-REV (2.5 μg), and pVSV-g (3 μg). After 48 h, human epidermal keratinocyte 293T cell supernatants containing target viruses were collected, filtered, and concentrated by ultracentrifugation. Collected viruses were aliquoted and supernatants containing target viruses were collected, filtered, and concentrated by ultracentrifugation. Collected viruses were aliquoted and stored at −80˚C. For viral titration, 2 × 105 HaCaT cells were seeded in 6-well plates, and then nontarget pLKO.1 nonspecific control shRNA (shNS) or target lentiviral particles were serially added with 8 μg/ml polybrene (Sigma-Aldrich). After 12 d, relative titers were determined by colony counting under purumycin (1 μg/ml) selection.

Transfection of HaCaT cells

Small interfering RNA (siRNA) transfection was performed, as described previously (17). Briefly, HaCaT cells were transfected with scrambled, nonspecific control siRNA (shNS), or siRNAs specific for hNOX4, hATG5, hATG7, hATG12, hATG16L1, and hATG13 (Thermo Shandon, Pittsburgh, PA). Negative control sections were treated with serum from nonimmunized rabbits (Dako) in place of primary Ab. Cells were fixed in 4% paraformaldehyde at 4˚C for 10 min. They were then permeabilized with 0.01% Triton X-100, before being blocked with 10% BSA for 1 h. Cells were stained with primary Ab (rabbit anti-human, diluted 1:400) and biotin-labeled secondary Ab (anti-rabbit IgG-tetramethylrhodamine isothiocyanate or anti-rabbit IgG Alexa488) for 1 h. Unbound Ab was removed with PBS, and cells were imaged using a Zeiss LSM510 META confocal microscope (Zeiss).

Transmission electron microscopy

Transmission electron microscopy was performed, as described previously (18). Briefly, cells were fixed in 4% paraformaldehyde at 4˚C for 10 min. They were then permeabilized with 0.01% Triton X-100, before being blocked with 10% BSA for 1 h. Cells were stained with primary Ab (rabbit anti-human, diluted 1:400) overnight, and then incubated with secondary Ab (anti-rabbit IgG-tetramethylrhodamine isothiocyanate or anti-rabbit IgG Alexa488) for 1 h. Unbound Ab was removed with PBS, and cells were imaged using a Zeiss EM 901 transmission electron microscope.

Immunohistochemistry

The subcellular localization of human p62 in tissue sections was determined through immunohistochemical staining, performed as described previously (17). Biopsy tissue obtained from patients with eczema and atopic dermatitis (AD), as well as five healthy controls, was fixed in formalin, embedded in paraffin, and sectioned to a thickness of 3 μm. After overnight incubation with the primary Ab (rabbit polyclonal p62 Ab, diluted 1:400; Abgent) at 4˚C, tissue sections were incubated with the substrate 3,3-diaminobenzidine (Dako) to generate a colored signal. They were lightly counterstained with Mayer’s hematoxylin and mounted using Immu-Mount (Thermo, Saint Louis, MO). Negative control sections were treated with serum from nonimmunized rabbits (Dako) in place of primary Ab. Cells displaying cytoplasmic staining were considered to be positive. A single pathologist examined all the immunostained tissue sections.

DNA and RNA extraction and RT-PCR analysis

The extraction of RNA from cells or paraffin-embbeded skin tissue and semi-quantitative RT-PCR were performed, as described previously (17). Briefly, total RNA was extracted using the TRIzol reagent (cells; Invitrogen) or High Pure RNA Paraffin Kit (tissue; Roche Applied Science, Mannheim, Germany). PCR reactions involved 35 cycles of annealing, extension, and denaturation. The annealing step consisted of 30 s at one of various annealing temperatures, as follows: 52˚C for p62/SQSTM1 and β-actin; 54˚C for Atg5; 58˚C for TLR2 and TLR4; 59˚C for IL-6; 63˚C for Beclin-1 and IL-6; and 66˚C for TNF-α. The following primers were used: p62/SQSTM1 forward 5′-CTGCCGACAGCTGATTGTG-3′ and reverse 5′-CTCACTTCAATGCCCAGAGG-3′; IL-6 forward 5′-CTGAGTGC-TCCTCTCACC-3′ and reverse 5′-GGTACAGAATGTCGCC-3′; NF-κB forward 5′-CAGAGGAAGATGTCCCGG-3′ and reverse 5′-CTTGTGCTGATAGAGG-3′; and reverse 5′-CCAGATTGCT- CTTGTGTTTCA-3′; TLR4 forward 5′-CTGCAATGATCAAGGAGC-3′ and reverse 5′-TCCACTCAGGTGTTCCG-3′ and reverse 5′-TCCACTCACGTAGATGTG-3′; β-actin forward 5′-ATCTGCGACACACTCTCCTAAGT-3′ and reverse 5′-A- CACCAACAGATGAGCAGAAAT-3′. Western blot, ELISA analysis, and NF-κB luciferase assays

Western analysis and cytokine ELISAs (for TNF-α and IL-6) were performed, as described previously (17). Briefly, NF-κB luciferase assay was also performed, as described previously (17). Briefly, HaCaT cells were transfected with a NF-κB-luc construct and siRNA (for HaCaT cells) or transduced with Ad5HSV-NF-κB-luc. Following stimulation with MALP-2 (100 ng/ml) for 24 h, cells were lysed and their luciferase activity was analyzed using the Luciferase Assay System (Promega, Madison, WI), according to the manufacturer’s instructions.

Immunofluorescence analysis

Immunofluorescence analysis for LC3 was performed, as described previously (18). Briefly, cells were fixed in 4% paraformaldehyde at 4˚C for 10 min. They were then permeabilized with 0.01% Triton X-100, before being blocked with 10% BSA for 1 h. Cells were stained with primary Ab (rabbit anti-human LC3, diluted 1:400) overnight, and then incubated with secondary Ab (anti-rabbit IgG-tetramethylrhodamine isothiocyanate or anti-rabbit IgG Alexa488) for 1 h. Unbound Ab was removed with PBS, and cells were imaged using a Zeiss LSM510 META confocal microscope (Zeiss).

Transmission electron microscopy

Transmission electron microscopy was performed, as described previously (18). Briefly, after MALP-2–stimulated human primary keratinocytes were fixed with 3% formaldehyde and 2% glutaraldehyde (Electron Microscopy Sciences, Road Hatfield, PA) in 0.1 M sodium cacodylate buffer (pH 7.4; Ted Pella, Redding, CA) for 1 h, they were then washed with 0.1% cacodylate buffer and postfixed with 1% osmium tetroxide (Electron Microscopy Sciences) and 0.5% potassium ferricyanide in cacodylate buffer for 1 h. Cells were then dehydrated with increasing concentrations of ethanol, and infiltrated with Epon-Araldite resin at 80˚C for 24 h. Ultrathin sections (70–80 nm) were obtained using an ultramicrotome (RMC MT6000-XL). Sections were counterstained with uranyl acetate and lead citrate and viewed using a Zeiss EM 902 electron microscope.

Immunohistochemistry

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Statistical analysis

All data are presented as the mean ± SD of independent experiments. Statistical analysis was performed using a paired Student t test with Bonferroni correction or, for multiple comparisons, ANOVA. The p values <0.05 were deemed to be statistically significant.

Results

TLR2/6 stimulation activates autophagy in primary human keratinocytes

It has previously been shown that TLR ligands stimulate the formation of autophagosomes and autophagolysosomes (7, 9, 10). However, it is not known whether TLR agonists activate autophagy in primary human keratinocytes. To determine whether TLR2/6 stimulation activates autophagy in human keratinocytes, we stimulated primary human keratinocytes with MALP-2 (100 ng/ml) and measured the formation of organelles displaying punctate LC3 staining (autophagosomes) using endogenous LC3 as an autophagic marker. As shown in Fig. 1A, MALP-2 induced autophagy in primary human keratinocytes, with peak activation occurring at 36 h. In addition, posttranslational modification of LC3-I, yielding LC3-II (19), was assessed in MALP-2–stimulated human keratinocytes. Significant increases in the LC3-II:LC3-I ratio were observed in primary human keratinocytes (Fig. 1B) and HaCaT cells (Supplemental Fig. 1A) after MALP-2 stimulation. LC3 autophagosome formation was significantly inhibited in cells pretreated with 3-MA or wortmannin, specific inhibitors of autophagy (20, 21) (Fig. 1C). Additionally, MALP-2–induced punctate LC3 staining was significantly reduced in cells in which Beclin-1 or Atg5 was silenced through transfection with a specific lentiviral shRNA (Fig. 1D). Transfection of primary human keratinocytes with shRNAs specific to hBeclin-1 (shBeclin-1) or hAtg5 (shAtg5) significantly decreased the expression of these genes relative to those transduced with a control shRNA (Fig. 1D, inset).

Colocalization of LC3 and lysosomes (the latter stained with LysoTracker) was significantly increased in primary human keratinocytes following MALP-2 stimulation (Fig. 1E). In primary keratinocytes, the ability of MALP-2 to increase the LC3-II:LC3-I ratio (measured by Western blotting) was attenuated by pretreatment with 3-MA (Fig. 1F). Furthermore, pretreatment with the vacuolar H+–ATPase inhibitor Baf-A1, which prevents lysosomal

![FIGURE 1. TLR2/6 stimulation activates autophagy in human epidermal keratinocytes. A, Immunofluorescence analysis of LC3 conversion. Primary human keratinocytes were stimulated with MALP-2 (100 ng/ml) for the indicated periods of time. Cells were stained with DAPI to visualize nuclei (blue), and immunolabeled with the combination of an anti-LC3 Ab and an Alexa 488-conjugated goat anti-rabbit IgG (green). Upper, Representative immunofluorescence images. Scale bar, 10 μm. Lower, Percentages of cells with LC3-positive punctae. B, Western blot analysis of LC3 and β-actin levels in primary human keratinocytes stimulated with MALP-2 (100 ng/ml) for the indicated periods of time. C and F, Primary human keratinocytes were stimulated with MALP-2 in the absence or presence of 3-MA (3MA; 2 h, 10 μM), wortmannin (WM; 1 h, 100 nM), or Baf-A1 (2 h, 200 nM), and subjected to immunofluorescence (C) or Western blot analysis (F). Cells treated with rapamycin (rapa; 4 h, 20 μg/ml) were used as a positive control. D, Primary human keratinocytes transfected with lentiviruses expressing shNS or shRNAs specific for Beclin-1 (shBeclin-1) or Atg5 (shAtg5) were cultured in the absence or presence of MALP-2 (100 ng/ml) for 36 h. Cells then were stained with anti-LC3–Alexa 488 (diluted 1:400), and the percentage of cells with LC3 punctae was determined. Transfection efficiency was assessed by RT-PCR (inset). E, MALP-2 induced colocalization of autophagosomes (endogenous LC3, red) and lysosomes (endogenous lamp-1, green) in primary human keratinocytes. Human primary keratinocytes were treated with MALP-2 for 48 h and subjected to immunofluorescence analysis. Representative images are shown. Scale bar, 10 μm. B and F, Gel images are representative of three independent replicates. The ratio of the intensities of the LC3-II and LC3-I bands is indicated below each lane. Data in A, C, and D represent the mean ± SD of three independent replicates, each comprising at least 250 cells scored in five random fields. **p < 0.01 (versus control). SC, solvent control (treated with 0.1% DMSO).](http://www.jimmunol.org/content/182/9/4549/F1.large.jpg)
acidiﬁcation and the fusion of autophagosomes with lysosomes (19, 22), signiﬁcantly increased the LC3-II:LC3-I ratio in MALP-2–stimulated cells (Fig. 1F). Using a transmission electron microscope, we analyzed numbers of autophagosome-like vacuoles with double-membrane structures (data not shown). As shown in Supplemental Fig. 1B, morphologic analysis of keratinocytes by transmission electron microscopy revealed the cytoplasmic accumulation of autophagic vacuoles. Thus, MALP-2 actively induces both autophagy and autophagosome–lysosome fusion in primary human keratinocytes.

**TLR2/6 stimulation induces p62 expression in human keratinocytes**

It was previously shown that IFN-γ and TLR signaling cooperate to stimulate late expression of p62 in murine macrophages (23). Additionally, TLR ligands induce the formation of autophagosomes and autophagolysosomes (7, 9, 10). However, it is not known whether TLR ligands induce p62 expression and/or activate autophagy in human keratinocytes. To address this, we stimulated primary human keratinocytes with MALP-2 and measured p62 mRNA and protein expression by RT-PCR and Western blotting, respectively. We further assessed the role of TLR2 in MALP-2–induced p62 mRNA expression. Transfection of HaCaT cells with a siRNA specific for hTLR2, but not one targeted to hTLR4, signiﬁcantly inhibited MALP-2–induced p62 expression (Fig. 2C). Thus, MALP-2 actively induces p62 expression in primary human keratinocytes via TLR2.

**NOX-dependent generation of ROS is required for the induction of p62 expression and activation of autophagy in TLR2/6-stimulated primary human keratinocytes**

ROS derived from superoxide play multiple roles in innate immune responses, including in microbial killing, the regulation of apoptosis, cytokine production, and gene expression, and the activation of antibacterial autophagy (24, 25). We ﬁrst examined whether MALP-2 increased the intracellular generation of superoxide in human keratinocytes using the oxidative ﬂuorescent dye dihydroethidium (DHE). As shown in Fig. 3A, MALP-2–induced superoxide production was detected within 30 min, peaking 1 h after stimulation. We next examined whether MALP-2–dependent ROS generation was required for p62 expression and the activation of autophagy. Treatment of keratinocytes with either the general ROS scavenger NAC or the NOX inhibitor DPI, but not the xanthine oxidase inhibitor allopurinol, signiﬁcantly and dose dependently attenuated MALP-2–induced p62 expression (Fig. 3B). Previously, it has been shown that HaCaT cells express signiﬁcant amounts of the gp91phox homologs NOX2 and NOX4, but only very low levels of NOX1 mRNA (26). Thus, we further investigated the roles of NOX2 and NOX4 in the MALP-2–mediated induction of p62. Silencing NOX2 or NOX4 using speciﬁc siRNAs signiﬁcantly attenuated MALP-2–induced p62 expression in HaCaT cells (Fig. 3C).

The MALP-2–induced formation of autophagosomes displaying punctate LC3 expression (Fig. 3D) and increase in the LC3-II:LC3-I ratio (Fig. 3E) was dose dependently abrogated by pretreatment with NAC or DPI, but not allopurinol. These data suggest that TLR2/6-dependent ROS generation also plays a role in the activation of autophagy in keratinocytes. Transfection of HaCaT cells with siRNAs speciﬁc to hNOX2 and hNOX4 signiﬁcantly inhibited autophagosome formation in response to MALP-2 (Fig. 3F). These data suggest that the NOX-dependent generation of ROS is critically involved in MALP-2–mediated p62 expression and autophagy in keratinocytes.

**TLR4 stimulation also leads to ROS-dependent activation of autophagy and p62 expression in keratinocytes**

Having established the role of TLR2/6 in the induction of autophagy, p62 expression, and ROS generation in human keratinocytes, we examined whether other TLR ligands function similarly in keratinocytes. Although previous studies showed that the TLR4 agonist LPS/endotoxin induced autophagy in macrophages (7, 9), a similar response has not been demonstrated in keratinocytes. As TLR4 was shown to be constitutively and inducibly expressed in HaCaT cells (2), we ﬁrst examined punctate LC3 expression and LC3-II levels in HaCaT cells after LPS stimulation. Treatment of HaCaT cells with LPS robustly increased punctate LC3 expression and LC3-II band intensities at 18 h, indicating activation of autophagy (Fig. 4A, 4B). The marked increase in punctate LC3 staining was largely abrogated by pretreatment with 3-MA (Fig. 4A), a novel inhibitor of autophagy activation (20, 21). TLR4/ LPS stimulation signiﬁcantly increased the endogenous expression of p62 and LL-37 in HaCaT cells (Fig. 4C, p62 and LL-37 mRNA; Fig. 4D, p62 protein). We further showed that LPS stimulation robustly activated ROS generation in HaCaT cells in a time-dependent manner, and that the peak was observed within 30 min of stimulation (Fig. 4E). Blocking ROS production using the antioxidants NAC and DPI dose-dependently inhibited LC3-II expression (Fig. 4F). Additionally, transfection of HaCaT cells...
with siRNAs specific for hNOX2 and hNOX4 significantly inhibited LPS-induced p62 expression (Fig. 4G). These data collectively show that the activation of TLR4, as well as TLR2/6, significantly upregulated NOX-dependent ROS generation, which contributes to autophagy activation and the expression of p62 and IL-37 in human keratinocytes.

**MyD88 and TRAF6 are required for p62 expression and the activation of autophagy in keratinocytes**

It was previously shown that the adaptor protein p62 selectively interacts with the TRAF domain of TRAF6 and plays an important intermediary role in the activation of NF-κB by TNF-α or IL-1 (27). Additionally, MyD88 plays a role in TLR4- or TLR7-induced activation of autophagy (7, 10). We therefore examined whether MALP-2–dependent activation of autophagy was mediated by MyD88 and/or TRAF6, both of which contribute to TLR signaling. When HaCaT cells were transfected with siRNAs specific for hMyD88 and hTRAF6, MALP2/6-dependent p62 protein (Fig 5A) and mRNA expression (Fig 5B) were significantly reduced. Furthermore, the MALP-2–induced increases in LC3-positive autophagosome formation (Fig 5C) and the LC3-II:LC3-I ratio (Fig 5D) were significantly reduced in HaCaT cells transfected with siRNAs specific for hMyD88 or hTRAF6. These data suggest that both MyD88 and TRAF6, key signaling intermediates in TLR-dependent signaling, are involved in the activation of autophagy in human keratinocytes.

The autophagy pathway negatively regulates TLR2/6- and/or TLR4-induced inflammatory responses and p62 expression in keratinocytes

Accumulating evidence suggests that autophagy contributes to the regulation of cellular inflammation (28). Recently, it has been reported that autophagy-linked genes are involved in susceptibility to Crohn’s disease (29), and that autophagy defects exacerbate endotoxin-induced activation of the inflammasome (30). We next examined whether the activation of autophagy plays a role in the regulation of inflammatory responses and NF-κB activation in primary keratinocytes. To test this, primary human keratinocytes were pretreated with 3-MA or wortmannin, two inhibitors of the kinase PI3K that is required for the activation of autophagy (31), or transfected with shBeclin-1 or shAtg5 prior to MALP-2 stimulation. Notably, the secretion of TNF-α and IL-6 was dose dependently increased in primary human keratinocytes pretreated with 3-MA or wortmannin (Fig. 6A). Primary human keratinocytes transfected with shBeclin-1 or shAtg5 displayed significant increases in MALP-2–induced production of the proinflammatory cytokines TNF-α and IL-6 (Fig. 6B). Additionally, MALP-2–dependent NF-κB reporter gene activity was significantly increased in HaCaT cells pretreated with 3-MA or wortmannin (Supplemental Fig 2A) and primary human keratinocytes transfected with shBeclin-1 or shAtg5 (Fig. 6C).

Next, the role of autophagy in regulating p62 expression was determined in human keratinocytes. Blockade of the autophagy pathway negatively regulates TLR2/6- and/or TLR4-induced inflammatory responses and p62 expression in keratinocytes.
pathway using 3-MA or wortmannin significantly increased MALP-2–induced p62 protein expression in primary human keratinocytes (Fig. 6D). Transfection of primary human keratinocytes with shBeclin-1 or shAtg5 significantly increased the induction of p62 protein expression following MALP-2 stimulation (Fig. 6E). We further characterized the role of autophagy in TLR4–stimulated keratinocytes. Consistent with the results of TLR2/6 stimulation, pretreatment of HaCaT cells with 3-MA or wortmannin significantly upregulated LPS-induced NF-κB p65 luciferase activity (Supplemental Fig. 2A), the secretion of TNF-α and IL-6 (Supplemental Fig. 2B), and p62 protein expression (Supplemental Fig. 2C). Similarly, higher levels of p62 protein were detected in LPS-stimulated HaCaT cells transfected with siRNAs specific for hbeclin-1 and hatg5 than in those transfected with a control siRNA (Supplemental Fig. 2D). Thus, the activation of autophagy contributed to the control of inflammatory responses through effects on NF-κB, but downregulated p62 accumulation, suggesting a negative role for autophagy in TLR2/6– and/or TLR4–induced inflammatory responses in human keratinocytes.

**TLR2/6– and/or TLR4–dependent p62 expression is required for the activation of inflammatory signaling and stimulation of keratinocyte proliferation**

Recent studies have defined the roles of p62 in NF-κB activation and oncogenesis in autophagy-defective tumor cells (16), suggesting its involvement in inflammatory responses and tumorigenesis. To further explore the role of p62 in inflammatory responses in human keratinocytes, p62 was knocked down in HaCaT keratinocytes through transfection with a specific siRNA. Silencing p62 in this way led to significant decreases in the expression of TNF-α and IL-6 mRNA and protein in response to MALP-2 stimulation (Fig. 7A, 7B). The induction of LL-37 mRNA expression in HaCaT cells by TLR2/6 was suppressed by transfection with siRNA specific to hp62 (si-p62; Fig. 7A).

Following TLR2/6 stimulation, the activation of MyD88-dependent TRAF6 signaling leads to the activation of TGF-activating kinase 1, which phosphorylates the IKK complex (32). The IKK complex, in turn, phosphorylates IκB proteins, resulting in their ubiquitination and degradation (32). This key event triggers the translocation of NF-κB to the nucleus, where it mediates the transcription of target genes (32). To further investigate the underlying mechanisms by which p62 modulates TLR2/6–dependent inflammatory responses, we examined whether p62 played a role in IKKα/β phosphorylation and IκBα degradation in response to MALP-2. As shown in Fig. 7C, MALP-2 stimulation led to the time-dependent phosphorylation of IKKα/β and concomitant degradation of IκBα in human primary keratinocytes transfected with shNS. The transduction of primary keratinocytes with shRNA specific for hp62/SQSTM1 prior to MALP-2 stimulation markedly inhibited the phosphorylation of IKKα/β and degradation of IκBα in human keratinocytes (Fig. 7C). Moreover, the silencing of p62 in HaCaT cells by p62 siRNA significantly inhibited NF-κB–luciferase reporter gene activity in response to MALP-2 treatment (Fig. 7D). We next examined whether the role of p62 in mediating inflammatory responses was confined to TLR2/6 responses. Silencing of p62 led to the significant inhibition of LPS-induced expression of TNF-α, IL-6, and LL-37, and NF-κB reporter gene activity, in HaCaT cells (Supplemental Fig. 3A–C). Thus, p62 appears to play
critical roles in TLR2/6- and/or TLR4-induced inflammatory responses through activation of NF-kB signaling.

We further investigated whether p62 plays roles in MALP-2–dependent cell proliferation, cyclin D1/Cdk4 expression, and the activation of Akt and ERK1/2 in human keratinocytes. As shown in Fig. 7E, MALP-2 stimulation resulted in a substantial increase in keratinocyte proliferation, as measured in a Cell Counting Kit-8 assay. Silencing p62 expression led to the time-dependent inhibition of keratinocyte proliferation (Fig. 7E). Additionally, transduction of human primary keratinocytes with shRNA specific for p62 efficiently reduced the expression of cyclin D1/Cdk4 and phosphorylation of Akt (Fig. 7F), suggesting that p62 plays key roles in keratinocyte proliferation and cell cycle progression. When primary keratinocytes were transduced with shRNA specific for p62, but not shNS, the phosphorylation of ERK1/2 was significantly reduced (Fig. 7G). These data collectively show that p62 plays important regulatory roles in keratinocyte inflammatory responses through the activation of IKK complex–NF-kB signaling, and in keratinocyte proliferation via cyclin D1-/Cdk4-, Akt-, and ERK1/2-dependent signaling pathways.

Epidermal expression of p62 and LL-37 is up-regulated in patients with psoriasis, but not AD

Based on the roles of p62 in the regulation of inflammatory responses and proliferation, we next extended our analyses to determine whether p62 levels are elevated in inflammatory skin dis-
orders. To test this hypothesis, we performed comparative analyses of epidermal p62 expression in plaque psoriasis and eczema. Psoriasis is characterized by increased inflammation and the release of antimicrobial peptides, as well as hyperproliferation caused by disturbed differentiation of keratinocytes (3, 33). Eczemas represent a group of etiological and regional inflammatory skin disease variants characterized by pruritic, papulovesicular, and weeping dermatitis (34). As eczemas are a diverse group of disorders, we focused on the p62 expression profile in AD, an inflammatory skin disease that is typically familial, and one that is associated with IgE reactivity, Th2 predominance, and mast cell hyperactivity (35, 36).

To examine the role of p62 and cathelicidin LL-37 in inflammatory skin conditions, we measured the expression of p62 and LL-37 in the epidermis of biopsy samples obtained from psoriasis and AD patients and healthy subjects. Immunohistochemical staining revealed high numbers of p62-expressing keratinocytes in the skin of all psoriatic patients studied (35 of 35), but in no AD patients (0 of 22) and only a minority of healthy controls (5 of 16). The p62 expression was primarily detected in the cytoplasm in samples of psoriatic skin (Fig. 8A). As shown in Fig. 8B, RT-PCR analysis of mRNA extracted from patient skin samples revealed significantly higher expression of p62 and LL-37 in psoriasis patients than in AD patients or healthy controls. These results indicate that lesional psoriatic skin displays marked upregulation of p62 and cathelicidin and may be involved in the pathogenesis of psoriasis in humans.

**Discussion**

In human keratinocytes, TLR signaling robustly activates autophagy and p62 expression. In cells in which autophagic function was lost, inflammatory responses were profoundly exacerbated. Expression of p62 increased in parallel with these changes. In MALP-2–treated cells, knockdown of p62 not only reduced pro-inflammatory cytokine production, but also inhibited proliferation. In psoriatic skin, but not in skin from AD patients, p62 expression was significantly increased. These findings suggest the involvement of autophagy in the regulation of excessive cutaneous inflammation and implicate the upregulation of p62 in the pathogenesis of psoriasis.

Accumulating evidence suggests that the recognition of multiple pathogen-derived molecules can activate autophagy, principally in mononuclear phagocytes (7, 9, 10). TLR7 and TLR4 ligands have been reported to be potent inducers of autophagy in macrophages (7, 9). Additionally, stimulation of TLR2 through zymosan treatment can induce autophagy, specifically the formation of phagosomes and their fusion with lysosomes (37). However, it has not been detailed previously whether TLRs activate autophagy in human keratinocytes. Our data provide evidence that TLR signaling robustly activates autophagy in human keratinocytes. Electron microscopic imaging showed the rapid formation of autophagosomes and autolysosomes in human keratinocytes following TLR2/6 stimulation. Moreover, cells stimulated with MALP-2 displayed clear autophagic features, including punctate LC3 staining and colocalization of LC3 and lysosome markers. In addition,
MALP-2 stimulation significantly increased levels of lipid-bound LC3-II. During autophagy, the autophagic marker LC3 is cleaved and covalently conjugated to phosphatidyl-ethanolamine, yielding LC3-II, which is specifically targeted to elongated, Atg12-/Atg5-associated autophagosome precursors, thereafter remaining associated with autophagosomes (38, 39). Moreover, our data show that TLR4/LPS stimulation further induces autophagy in keratinocytes (see Fig. 4). Thus, our data suggest that the stimulation of innate immunity-associated TLRs in primary human keratinocytes actively induces autophagosome formation and regulates the maturation of autophagolysosomes.

We found p62 expression and the activation of autophagy to be dependent on NOX-mediated ROS generation and MyD88- and TRAF6-mediated signaling. Indeed, p62 mediates the TNF-α- and IL-1-dependent activation of NF-κB by interacting with the specific adapters receptor-interacting protein and TRAF6 (27, 40). Recent studies demonstrated that ROS generation contributes to the activation of autophagy, TLR activation, and phagocytosis in PMA-stimulated neutrophils (41). Similar to a previous study (25), we found that NOX activity and ROS generation played important roles in the activation of autophagy in primary human keratinocytes. Moreover, MyD88 has been previously implicated in TLR4- or TLR7-induced autophagy (7, 9, 10). Both key adaptor molecules in TLR signaling, MyD88 and TIR-domain–containing adapter-inducing IFN-β, modulate the interaction between Bcl-2 and Beclin-1, thereby actively triggering and regulating autophagy (10). Together, these data implicate innate immune signaling and key adaptor molecules in the activation of autophagy pathways, not only in leukocytes, but also in nonleukocytic cells.

In this study, we have demonstrated that the inhibition of autophagy results in significantly increased inflammatory cytokine production and p62 expression in primary human keratinocytes. Enhanced inflammatory responses and increased cell proliferation were observed in keratinocytes treated with compounds that interfere with early (3-MA) and late (Baf-A1) autophagic processes or with siRNA targeted to genes essential for autophagy (hBeclin-1, hAtg5). Recently, it was shown that a defect in autophagy potentiated RIG-like receptor signaling and increased IFN secretion caused by ROS generated by dysfunctional mitochondria (42).

Evidence suggests that autophagy acts to regulate cellular homeostasis and signaling by altering the availability of key adaptor proteins, such as p62 (43) and Nbr1 (12). Notably, p62 is itself an important intracellular target for autophagy: its cytosolic accumulation represses inclusion body formation (43). Previous studies showed that the inhibition of autophagy leads to the accumulation of p62 in transformed cells, thus compromising the clearance of ubiquitinated proteasomal substrates (44). Together, these data suggest that the pathways controlling the activation of autophagy tightly control p62 levels to prevent homeostasis from being disrupted during inflammatory responses.

Our data further show that knockdown of p62 using a specific siRNA reduced the expression of inflammatory cytokine genes and cathelicidin, cell proliferation (as assessed by measuring cyclin D expression), and NF-κB promoter activity in keratinocytes. Earlier studies showed that dominant-negative mutants and the downregulation of p62 using an antisense plasmid markedly inhibited the activation of NF-κB by TNF-α, suggesting a critical role for p62 (40). It has been reported that cytosolic p62/SQSTM1 expression is induced by Ras, is required for IκB kinase activation through polyubiquitination of TRAF6, and contributes to the development of Ras-induced lung adenocarcinomas in murine models (45). Additionally, sustained p62 expression accompanying defective
autophagy results in altered NF-κB regulation and gene expression, thus favoring tumorigenesis (16). As an atypical protein kinase C scaffold protein, p62 plays an important role in the activation of NF-κB in response to IL-1, TNF-α, and receptor activator for NF-κB ligand through the assembly of an aPKC/p62/TRAF6 multi-protein complex (46). Notably, our data demonstrate that p62 expression is crucial for the TLR-dependent activation of IKKα/β and degradation of IκBα in human keratinocytes (see Fig. 7). Thus, the cytosolic accumulation of p62/SQSTM1 may trigger TLR-induced inflammatory responses in keratinocytes through modulation of IKK–NF-κB signaling.

Both psoriasis and AD are common chronic and relapsing inflammatory skin diseases (33–35). Psoriasis is an inflammatory skin disease characterized by increased levels of TLRs 1, 2, 4, 5, and 9 (reviewed in Ref. 3). It has been suggested that upregulated TLR expression in psoriatic skin contributes to disease pathophysiology by increasing the local production of antimicrobial peptides, and stimulating cutaneous immune responses and epidermal hyperproliferation (3, 33). In contrast, monocytes from AD patients displayed significantly impaired TLR2-mediated production of the proinflammatory cytokines IL-1β and TNF-α (47). Additionally, the immunologic factors involved in the pathogenesis of AD are associated with upregulated IgE reactivity, and increased numbers of mast cells, eosinophils, and CD4-activated Th2 cells (34–36). Importantly, we found that the expression of p62 and cathelicidin was higher in psoriatic skin lesions than in lesions from AD patients or skin from healthy controls (see Fig. 8). The current data partly corroborate the findings of previous studies, which showed that psoriatic skin contained high amounts of the antimicrobial peptide cathelicidin/LL-37 (48). LL-37 potently activates plasmacytoid dendritic cells to produce type 1 IFNs by binding nonstimulatory self-DNA and causing it to adopt an aggregated and condensed structure (49). Several previous studies further showed that levels of various antimicrobial peptides, including LL-37 and human β-defensin 2, are significantly decreased in acute and chronic lesions from AD patients (50). The depressed expression of innate immune effectors and antimicrobial peptides may contribute to the increased susceptibility of AD patients to bacterial and viral infections (3, 51, 52). Together, p62 and cathelicidin may play important roles in the homeostatic regulation of cutaneous inflammatory responses, especially in psoriasis. Future studies should clarify the detailed molecular mechanism(s) by which enhanced p62 expression exacerbates psoriatic inflammatory responses by breaking innate tolerance and driving autoimmunity.

The current findings demonstrate a major role for autophagy in the regulation of keratinocyte inflammation and provide new insights into the mechanisms by which TLR signaling activates autophagy in human keratinocytes. TLR innate signaling activation in keratinocytes triggers a simultaneous induction of innate inflammatory responses and autophagy pathways. Importantly, the autophagy pathways were found to play a key role in fine tuning of inflammatory responses in keratinocytes. The ability of the inhibition of keratinocyte autophagy to increase inflammation may have important implications for the development of novel therapeutic strategies for improving inflammatory homeostasis in psoriasis patients.

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